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Caloric dose-responsive genes in blood cells differentiate the metabolic status of obese men☆,☆☆,★

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Abstract

We have investigated the postprandial transcriptional response of blood cells to increasing caloric doses of a meal challenge to test whether the dynamic response of the human organism to the ingestion of food is dependent on metabolic health.

The randomized crossover study included seven normal weight and seven obese men consuming three doses (500/1000/1500 kcal) of a high-fat meal. The blood cell transcriptome was measured before and 2, 4, and 6 h after meal ingestion (168 samples). We applied univariate and multivariate statistics to investigate differentially expressed genes in both study groups.

We identified 624 probe sets that were up- or down-regulated after the caloric challenge in a dose-dependent manner. These transcripts were most responsive to the 1500 kcal challenge in the obese group and were associated with postprandial insulin and oxidative phosphorylation. Furthermore, the data revealed a separation of the obese group into individuals whose response was close to the normal weight group and individuals with a transcriptional response indicative of a loss of metabolic flexibility.

The molecular signature provided by the postprandial transcriptomic response of blood cells to increasing caloric doses of a high-fat meal challenge may represent a sensitive way to evaluate the qualitative impact of food on human health.
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Keywords: Gene expression; Dose–Response; High-fat meal; Insulin; Oxidative phosphorylation

1. Introduction

Many genetic and environmental factors are responsible for the development of obesity, including a changing food environment facilitating the consumption of energy-dense/high-fat diets, which are strongly and positively associated with overweight [1]. Therefore, one current focus of nutrition research is to understand how metabolic, physiological and genetic processes globally differ between health and disease, in particular, obesity [2].

Irrespective of whether a person is obese (OB) or has a normal weight (NW), food intake stresses the organism, shifting it constantly between fasting and postprandial conditions. The scientific

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☆☆ The study is registered at http://www.clinicaltrials.gov, NCT01995110.
★ Gene expression data are deposited in Gene Expression Omnibus (GEO, www. http://www.ncbi.nlm.nih.gov/geo/) and can be retrieved using the code GSE56960.

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community made use of this phenomenon to discuss the concept of "dysmetabolism" in the context of the postprandial response [3–10]. In this model, subjects with a physiologically fit metabolism increase their metabolic activity after the consumption of a meal challenge but efficiently return within a few hours in direction of the baseline activity — their metabolism thus reacts in a flexible manner to food ingestion. In contrast, subjects with an impaired metabolism, such as metabolically unhealthy OB people, may react less flexibly to the ingestion of meals, the control mechanisms responsible for returning to the baseline value being inefficient [3] — they can be regarded as metabolically inflexible. The value of this model was recently illustrated by the work of Kardinaal *et al.* [6] showing, with a set of 61 selected markers, that men suffering from the metabolic syndrome are characterized by an impaired phenotypic flexibility after consuming a high-fat challenge of ~3200 kcal. Metabolic inflexibility had also been previously observed in insulin-resistant individuals in the form of slow responses in gene and protein expression to changes in nutrient load [5,7,11].

Alterations in metabolic flexibility can thus be identified by conducting a dynamic evaluation of the postprandial response of subjects to a meal challenge [6]. However, the question of what caloric dose is necessary to identify alteration in the metabolic response of subjects has not been addressed until recently [12]. Indeed, particular attention was made to the study design of Schwander *et al.* [12,13] on which this transcriptomic report is based. Using a set of clinical chemistry parameters covering metabolic, inflammatory and hormonal processes, these authors investigated the caloric dose-dependent effect of a high-fat meal on the postprandial response of NW and OB subjects. This study showed that insulin could clearly differentiate NW from OB subjects at each of the three-caloric doses studied. However, the ability of the other parameters to differentiate the two study groups was specifically dependent on the choice of the caloric dose of the high-fat meal challenge. These results indicate that challenge studies may lead to wrong interpretations of the metabolic status of a subject, if the dose–response dimension is not considered.

In the past, traditional nutritional research strongly relied on classical clinical parameters such as insulin, glucose or blood lipid profiles to identify metabolic dysfunctions. In order to achieve holistic insights into the metabolic pathways mediating these processes, technologies like transcriptomics or metabolomics, which provide high-coverage data, are now increasingly being used. In this regard, the informative content of the report by Schwander *et al.* [12] was limited to a small set of clinical chemistry parameters. To obtain deeper mechanistic insights into the postprandial metabolic changes taking place in NW and OB subjects after the consumption of increasing caloric doses of a high-fat meal, we now report data obtained with the blood cell transcriptome of seven NW subjects and seven OB subjects randomly chosen from the primary study [12].

2. Material and methods

2.1. Study

The study assessed the postprandial kinetics of gene expression in blood cells of NW and OB subjects after consumption of three caloric doses of a high-fat meal. The study was conducted at the University Hospital Bern, Switzerland. The clinical chemistry parameters of the whole cohort have been published elsewhere [12]. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. Approval for the study was obtained from the Ethics Committee of the Canton Bern KEK number 006/11. All participating subjects provided informed consent.

2.1.1. Study design

In the crossover study design, each subject had to consume, in random order, three different caloric doses of a high-fat meal (500, 1000 and 1500 kcal) with at least 1 week between each meal. Blood was collected with an indwelling peripheral venous catheter from overnight fasted subjects prior to the consumption of each test meal, as well as at three (2, 4, 6 h for gene expression) or four time points (1, 2, 4, 6 h for clinical chemistry) after the ingestion of the test meal. For 6 h after test meal consumption, the subjects were not allowed to consume any additional foods or beverages except L of water.

2.1.2. Subjects

NW [body mass index (BMI) 20–25 kg/m^2, waist circumference<94 cm] and OB (BMI>30 kg/m^2>102 cm) male subjects, between 25 and 55 years, were recruited from the region Bern, Switzerland. Whole blood was collected from seven subjects of each group. The sample size of seven subjects was selected based on a previous postprandial crossover study of six healthy male individuals having ingested dairy breakfasts with an energy content of approximately 350 kcal. This caloric dose and number of subjects has been shown to be sufficient to identify statistically significant and biologically meaningful postprandial changes in over 500 transcripts expressed in blood cells [14].

2.1.3. Test meals

The high-fat meals consisted of plain bread, palm fat, salami and boiled eggs, obtained from Swiss supermarkets. The three high-fat meals had the same macronutrient composition with 61% of the energy coming from fat, 21% from carbohydrates and 18% from protein and differed only in their energy content, that is, 500, 1000 and 1500 kcal. Two hundred, 400 and 600 ml of Vittel water had to be drunk during consumption of the first, second and third meal, respectively.

2.2. Clinical chemistry

Blood was collected for genome-wide gene expression analysis and for evaluating the impact of meal consumption on clinical parameters including total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, endotoxin, glucagon–like peptide 1 (GLP-1), high-sensitivity C-reactive protein (CRP), glucose and insulin as described previously [12].

2.3. Processing of blood samples for gene expression analysis

2.3.1. Blood sampling

RNA for the microarray experiments was isolated from 168 whole blood samples taken before (0 h, fasting condition) and 2, 4 and 6 h after the consumption of each of the three different high-fat meals by seven NW and seven OB subjects. Whole blood was collected in PAXgene blood RNA tubes (2×2.5 ml, PreAnalytiX GmbH, CH, USA) and left at room temperature overnight. Afterwards, the PAXgene tubes were frozen at −80°C until further treatment.

2.3.2. RNA processing

Total RNA was extracted using PAXgene Blood RNA Kit (Qiagen, CH) according to the manufacturer's recommendations. In a second step, total RNA was purified and concentrated with RNeasy MinElute Cleanup Kit (Qiagen, CH). The quantity and quality of total RNA was measured with a NanoDrop spectrophotometer (NanoDrop, USA) and Agilent 2100 Bioanalyzer (Agilent Technology, USA). The next treatment included the depletion of globin mRNA with the GLOBINclear™ hybridization capture technology (Ambion, USA) according to the recommendations of the manufacturer. Genome-wide transcript profiling was performed with HG-U219 oligonucleotide expression probe arrays (Affymetrix, USA), targeting more than 36,000 transcripts. Before the sample hybridization on the Affymetrix 3′-expression array strips, globin-removed RNA underwent reverse transcription to synthesize first-strand cDNA, which was then transformed into a double-stranded DNA template for the transcription process. *In vitro* transcription synthesized amplified RNA (aRNA) and incorporated a biotin-conjugated nucleotide. The aRNA was then purified to remove salts, enzymes, unincorporated NTPs and inorganic phosphate. Finally, the biotin-labeled aRNA was fragmented and hybridized onto the array strip. After washing and staining steps, the arrays were measured with the imaging station of the Affymetrix GeneAtlas™ System. All experiments were performed according to the manufacturer's recommendations and protocols.

2.4. Statistical methods

2.4.1. Baseline characteristics of NW and OB subjects

Baseline differences of clinical chemistry parameters between NW and OB subjects were assessed with Mann–Whitney *U* test using the SYSTAT software (Version 13, Systat Software Inc., CA, USA). Differences were considered as significant when $P \leq .05$.

2.4.2. Processing of gene expression data

Affymetrix Human Genome 219 raw data were processed by RMA (Robust Multichip Average) using the limma R package (Version 3.26.9) to perform background correction, log2 transformation and quantile normalization of gene expression measures [15]. Exploratory analyses revealed the presence of an unknown confounding factor in the data. To address this problem, we took advantage of replicate samples (three baseline samples for each participant), since changes among replicates are caused by unwanted variation factors. All replicates were used to estimate W, a matrix of k unwanted variation factors as described in Jacob *et al.*, 2016 [16]. W was applied to the full dataset following the naïve RUV-2 (Removal of Unwanted Variation) approach [17] with three factors of variation (k=3) to produce sufficient correction. All subsequent analyses are based on corrected data.

2.4.3. Subtraction of baseline expression

For each participant and meal, we computed the difference between the expression profile of postprandial samples (t=2, 4 or 6 h) and the expression profile of the corresponding baseline sample (t=0). These differences, interpretable as $\log_2$-fold expression changes ($\log_2$FC) from baseline, were used for the subsequent analysis.

2.4.4. Measurement of the postprandial response with the incremental area under the curve (iAUC)

To summarize the postprandial gene expression changes for a given probe set in a given subject for a given meal, we defined the incremental area under the $\log_2$-fold change curve (iAUC). In analogy to the routine clinical use of the AUC of postprandial glucose as a risk factor, the AUC approach integrates the quantity of the gene expression signal over time. The iAUC analysis offers the advantage, over an analysis of individual postprandial time points, to deliver a robust, biologically meaningful picture of postprandial gene expression. Indeed, the amount of protein that can be expressed by a specific mRNA obviously depends both on its expression level and its duration of expression as recently discussed in detail by Palumbo *et al.* [18]. In addition, shifts in the kinetics of the postprandial response due to treatment effects and intraindividual variability in the digestive process might complicate the analysis of the data by driving the study results toward a level of detail that would be difficult to interpret. In that context, in addition to simplifying the data, the iAUC approach can more robustly cope with such changes as it purposely minimizes the impact of moderate kinetic shifts on the study results.

The continuous $\log_2$FC curve was obtained by linearly interpolating the three $\log_2$FC values (relative to baseline) at time points 2, 4 and 6 h. The iAUC was defined as the area under the resulting curve, computed using the trapezoidal rule by means of the caTools R package (Version 1.17.1) ('postprandial response': $iAUC_{NW,500}$, $iAUC_{NW,1000}$, $iAUC_{NW,1500}$, $iAUC_{OB,500}$, $iAUC_{OB,1000}$, $iAUC_{OB,1500}$). We further used the R package limma (Version 3.26.9) to calculate moderated t-statistics, *P*-values and false discovery rates (FDR) by fitting a linear model by generalized least squares (GLS) to allow for correlation between expression arrays from the same subjects.

The postprandial response of the clinical parameters (net iAUC) was calculated as described previously [12].

2.4.5. Identification of differential caloric-responsive genes

We used the R package limma (Version 3.26.9) to calculate differences in iAUC between meals (log fold change (LFC) of iAUC), moderated t-statistics, *P*-values and FDR by fitting a linear model by GLS ('caloric-response': $iAUC_{NW,1000-500}$, $iAUC_{NW,1500-1000}$, $iAUC_{NW,1500-500}$, $iAUC_{OB,1000-500}$, $iAUC_{OB,1500-1000}$, $iAUC_{OB,1500-500}$). Probe sets with LFC of $|iAUC| > 2$ and $P \leq .05$ (corresponding to 20% FDR) in at least one of the six meal comparisons were selected as "caloric-responsive probe sets" for subsequent analyses. The average variance of caloric-responsive genes was computed in NW and in OB group, and the difference of variance between the two groups was assessed with Wilcoxon-signed rank test. The term *caloric-responsive probe sets* denotes that the transcripts on the microarray probed by these probe sets responded in terms of decreased or increased levels of expression. This term will be used throughout the text.

2.4.6. Correlation of caloric-responsive genes with clinical chemistry parameters

Spearman rank correlation coefficients were calculated with R in order to identify caloric-responsive probe sets whose postprandial regulation significantly correlates with postprandial clinical chemistry parameters in the NW and OB subjects ($P \leq .05$). For this purpose, we used the net iAUC of clinical chemistry parameters and the iAUC of each caloric-responsive probe set. Two correlation analyses were conducted: (a) seven NW subjects at each of the three-caloric conditions and (b) seven OB subjects at each of the three-caloric conditions. Glucose was excluded from the correlation analyses as the first postprandial time point investigated for gene expression, that is, 2 h, was collected too late to detect the major postprandial changes in glucose metabolism that take place 15–30 min after consumption of meals.

2.4.7. Multivariate analyses of caloric-responsive genes

Unsupervised principal component analysis (PCA) was applied to the matrix of caloric-responsive probe sets (iAUC and individual time points) using SIMCA software Version 13.0 (Umetrics, Sweden). Based on the PCA of the iAUC data, metabolically active (MA) and metabolically normal (MN) subjects were defined. We further conducted supervised orthogonal partial least squares-discriminant analysis (OPLS-DA) with SIMCA software with the caloric-responsive probe sets of the 42 samples (14 subjects, 3 meals). Pareto scaling was applied on the data, the OPLS-DA model was tested with a sevenfold cross-validation and the corresponding Q^2 and R^2 values were used to assess the robustness of the model. Subsequently, the variable importance parameter values (VIP-values) were calculated by SIMCA to identify probe sets that contribute the most to the separation of volunteers. Probe sets with VIP-values>1.0 were used for further functional analysis.

2.4.8. Functional analyses of caloric-responsive genes differentiating MA from MN and NW from OB subjects

The GeneGO MetaCore™ software (Version 6.26, Thomson Reuters, GeneGO Inc., USA) [19] was used to identify pathways with significant enrichment of VIP-value selected caloric-responsive probe sets. Metacore is an integrated software suite for functional analysis of microarray data, among others. There is no peer-reviewed publication available describing the details of the Metacore pathway analysis method. Mitrea *et al.* [20] made an attempt to uncover some of these details and stated that Metacore uses two types of proprietary knowledge which includes canonical pathways and interaction database. The software applies centrality measures or a variation of these measures to score nodes in a given pathway. Centrality measures such as degree centrality, betweenness or eigenvector centrality describe the importance of a node relative to all other nodes in a network. Metacore uses a measure similar to node betweenness in order to score genes. Our gene lists consisting of probe sets from the OPLS-DA analysis with a VIP-value higher than 1.0 were uploaded to the software. Thresholds for pathway mapping were predefined by the system and background list was set to HG-U219. To account for multiple hypothesis testing, we extracted only pathways with FDR<0.05. Genes being enriched in these pathways were tested for their postprandial up- or down-regulation (using mean iAUC and testing its difference from 0 with Wilcoxon-signed rank test, $P \leq .05$).

The function of the genes that also significantly correlated with insulin and HDL was investigated with a pathway analysis using the MetaCore software.

A functional analysis of the caloric-dependent probe sets significant in each of the six groups $NW_{1500-1000}$, $NW_{1500-500}$, $NW_{1000-500}$, $OB_{1500-1000}$, $OB_{1500-500}$ and $OB_{1000-500}$ was also conducted with MetaCore, separating up-regulated genes from down-regulated genes.

Finally, the BMI-dependent genes proposed by Homuth *et al.* [21] to be signatures for attenuated insulin signaling (251 genes), erythrocyte to reticulocyte ratio shift (168 genes) and reduced defense against oxidative stress (62 genes) were inspected for their expression values throughout the six groups of the NW and OB subjects (NW_{500}, NW_{1000}, NW_{1500}, OB_{500}, OB_{1000}, and OB_{1500}).

2.4.9. CellMix test for estimating blood cell composition

The proportion of white blood cells in each sample was predicted with CellMix gedBlood function [22] using 17 gene signatures of blood immune cells defined in Supplementary Table 1 of Abbas *et al.* [23]. The method was applied to raw expression data according to the developer's descriptions. The significance levels for postprandial differences in cell composition were evaluated with Mann–Whitney *U* test ($P \leq .05$).

3. Results

3.1. Clinical chemistry of NW and OB subjects

To conduct gene expression analysis, we selected seven NW and seven OB subjects randomly from the main cohort [12]. Their baseline characteristics are summarized in Table 1. As for the main cohort, statistically significant differences in parameters related to obesity were observed between the two groups, including increased weight, BMI, waist circumference, glucose, insulin, triglycerides, ratio total cholesterol/HDL cholesterol and decreased HDL-cholesterol in the OB group. The postprandial responses (net iAUC) of clinical parameters for each participant after each caloric dose of the high-fat meals are listed in Suppl. Table 1.

3.2. Identification of differential caloric-responsive genes

We first extracted probe sets whose postprandial expression |iAUC| was above 2 and was significantly different ($P \leq .05$) in at least one of

Table 1
Basic characteristics of studied population group

	NW subjects (n=7)	OB subjects (n=7)
Age (year)	45.3±9.8	42.4±8.7
Height (cm)	178.3±6.4 *	175.0±7.5
Weight (kg)	75.2±8.3 *	129.3±11.1
BMI (kg/m^2)	23.6±1.5 *	42.2±2.9
Waist circumference (cm)	84.7±5.7 *	131.6±7.4
Glucose (mmol/L)	4.9±0.4 *	5.3±0.7
Insulin (mU/L)	4.3±1.7 *	19.4±6.3
Triglyceride (mmol/L)	0.8±0.3 *	2.1±1.2
Total cholesterol (mmol/L)	5.2±0.7 *	6.1±0.9
HDL-cholesterol (mmol/L)	1.6±0.2 *	1.2±0.3
Ratio cholesterol/HDL	3.2±0.5 *	5.4±1.4
CRP (mg/L)	0.6±0.6 *	2.7±2.0
Endotoxin (EU/ml)	2.2±0.4	3.0±1.7
GLP-1 (pmol/L)	20.2±9.0 *	49.7±23.3

Data are presented as means ± S.D. Mann–Whitney *U* test was used to determine significant differences between NW and OB subjects.
* Differences were considered as significant when $P \leq .05$.

the six comparisons of the caloric response ($iAUC_{NW,1000-500}$, $iAUC_{NW,1500-1000}$, $iAUC_{NW,1500-500}$, $iAUC_{OB,1000-500}$, $iAUC_{OB,1500-1000}$, $iAUC_{OB,1500-500}$). The *P*-value of ≤0.05 corresponds to an FDR-value of 20%. Though being high, this FDR cutoff is acceptable as nutritional studies tend to produce small signals and have high variability.

A total of 624 probe sets from the 49,386 measured probe sets fulfilled these criteria and were selected for subsequent analyses (see Suppl. Table 1). Fig. 1 graphically shows the postprandial regulation of each of these probe sets for the NW and OB subjects at each of the three caloric doses of the high-fat meal (upper panel) as well as their caloric response (lower panel). A large fraction (32%) of the 624 probe sets were significantly up-regulated (iAUC>2) in OB subjects having consumed 1500 kcal ($iAUC_{OB,1500}$). This behavior is reflected by the caloric comparisons, $iAUC_{OB,1500-1000}$ and $iAUC_{OB,1500-500}$ showing larger effects than $iAUC_{OB,1000-500}$. In contrast, NW subjects showed lower postprandial and caloric responses. Furthermore, we computed the variance of the 624 regulated genes in NW and OB subjects and observed a higher variance in OB (mean=6.2) in comparison to NW (mean=2.4) subjects (*P*<.001).

3.3. Correlation of caloric-responsive genes with clinical chemistry parameters

In order to investigate if there is a link between the postprandial regulation of the caloric-responsive genes and postprandial clinical chemistry parameters, we calculated Spearman rank correlation coefficients on the set of 42 samples (separately for NW and OB subjects) (Fig. 2). The significant correlation coefficients for the NW subjects range between 0.43 and 0.74 for positive correlations and between −0.43 and −0.72 for negative correlations. The significant correlation coefficients for the OB subjects range between 0.43 and 0.78 for positive correlations and between −0.44 and −0.76 for negative correlations (*P*≤.05). The number of caloric-responsive probe sets with significant correlation coefficients differs notably between the two groups of subjects; whereas, hardly any positive or negative correlations occur for the NW subjects, many correlations appear between probe sets and clinical chemistry parameters for the OB subjects. In particular, insulin is the clinical chemistry parameter with the highest correlation coefficient and the largest number of significant correlations (OB subjects: 216 probe sets with positive correlation and 66 probe sets with negative correlation; NW subjects: 20 probe sets with positive correlation and 11 probe sets with negative correlation). HDL also showed a large number of significant correlations for the OB subjects (OB subjects: 173 probe sets with positive correlation and 69 probe sets with negative correlation; NW subjects: 21 probe sets with positive correlation and 18 probe sets with negative correlation). In the OB subjects, insulin and HDL share 127 probe sets with significant correlation coefficients (101 positive and 26 negative).

The highest number of probe sets with significant correlation coefficients in the group of NW subjects is present for endotoxin (OB subjects: 6 probe sets with positive correlation and 4 probe sets with

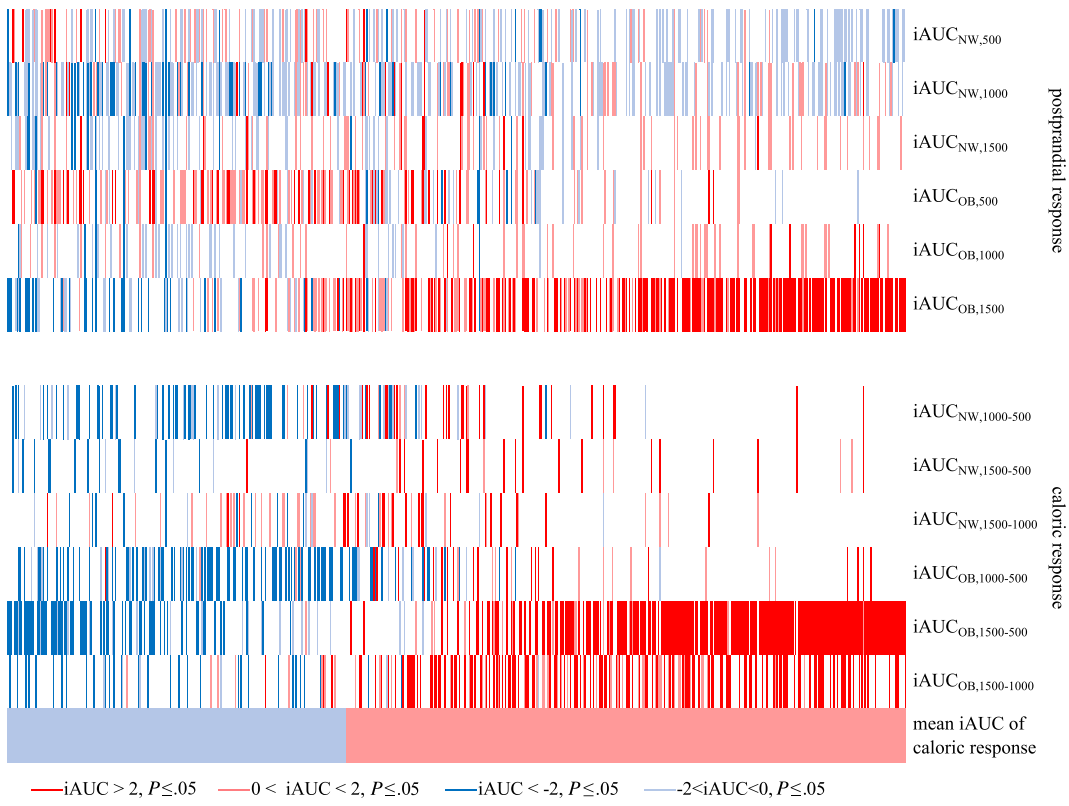

Fig. 1. Postprandial (upper panel) and caloric (lower panel) regulation of the caloric-responsive probe sets for the NW and obese OB subjects. The behavior of the 624 probe sets that showed a statistically significant (*P*≤.05, corresponding FDR 20%) difference in iAUC between two caloric doses of the high-fat meal with a magnitude of at least two in at least one of the six caloric comparisons ($iAUC_{NW,1000-500}$, $iAUC_{NW,1500-1000}$, $iAUC_{NW,1500-500}$, $iAUC_{OB,1000-500}$, $iAUC_{OB,1500-1000}$, $iAUC_{OB,1500-500}$) is presented in the lower panel. The upper panel illustrates the postprandial response ($iAUC_{NW,500}$, $iAUC_{NW,1000}$, $iAUC_{NW,1500}$, $iAUC_{OB,500}$, $iAUC_{OB,1000}$, $iAUC_{OB,1500}$) of the same probe sets. In both panels, the colors indicate the following characteristics: dark blue: *P*≤.05 and iAUC<−2, light blue: *P*≤.05 and −2 < iAUC<0, dark red: *P*≤.05 and iAUC>2, light red: *P*≤.05 and 0 < iAUC<2. For the upper panel, "iAUC" refers to the postprandial values. For the lower panel, iAUC refers to the caloric response, that is, to the change in expression between two caloric doses. Genes in both panels are ranked according to the mean caloric response (iAUC) over all six conditions. The probe sets (in columns) are in the same order for both panels.

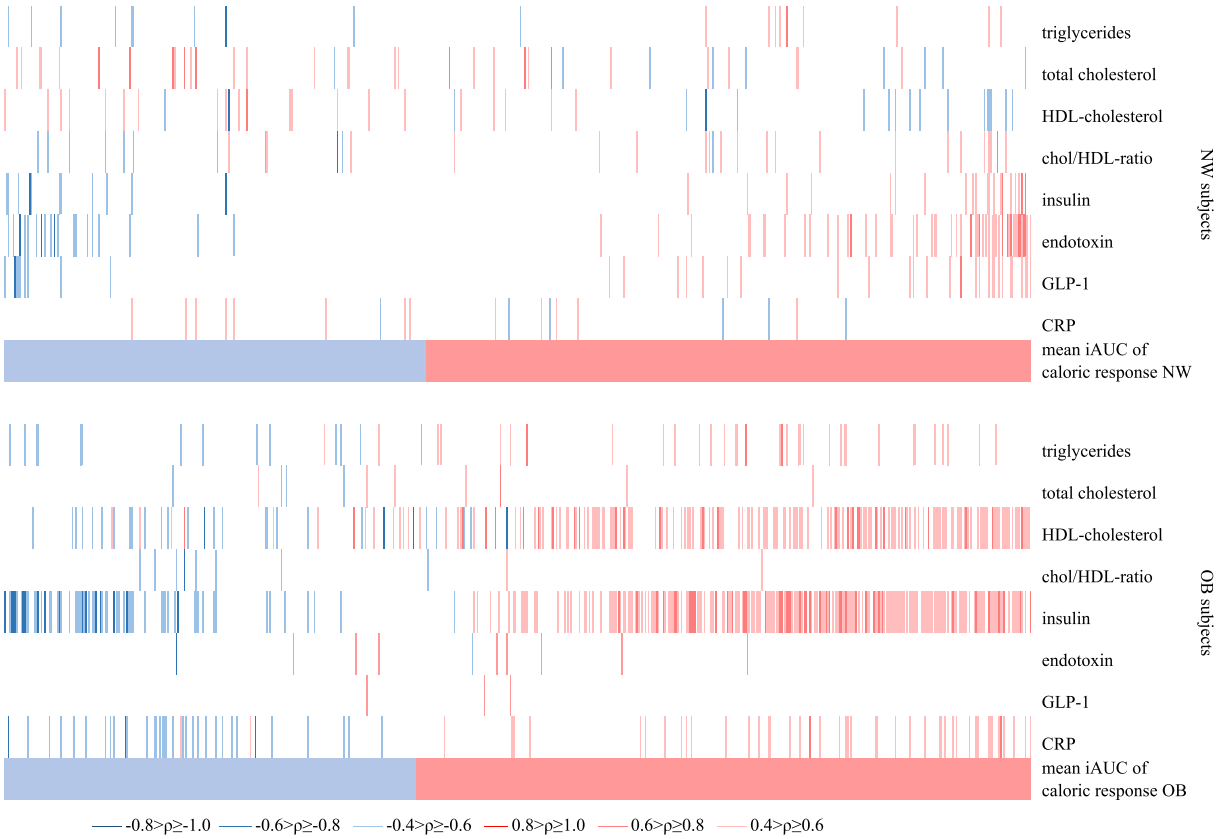

Fig. 2. Spearman's rank correlation coefficients of clinical chemistry parameters and expression of caloric-responsive probe sets. The 624 caloric-responsive probe sets are ranked according to the mean of the three conditions of the postprandial response of NW subjects ($iAUC_{NW,500}$, $iAUC_{NW,1000}$, $iAUC_{NW,1500}$) (upper panel) and OB subjects ($iAUC_{OB,500}$, $iAUC_{OB,1000}$, $iAUC_{OB,1500}$) (lower panel). The colors indicate the following Spearman's correlation coefficients ($P \leq .05$): dark blue: $-0.8 > \rho \geq -1.0$, medium blue: $-0.6 > \rho \geq -0.8$, light blue: $-0.4 > \rho \geq -0.6$, dark red: $0.8 > \rho \geq 1.0$ medium red: $0.6 > \rho \geq 0.8$ light red: $0.4 > \rho \geq 0.6$.

negative correlation; NW subjects: 51 probe sets with positive correlation and 19 probe sets with negative correlation). Of note, the postprandial net iAUCs for endotoxin are mainly positive among the subjects (see Suppl. Table 1 for details).

As the strongest and most significant correlations between the caloric-responsive probe sets and clinical chemistry parameters appeared for insulin in OB subjects, a functional analysis of the group of 216 caloric-responsive probe sets that positively correlated with this parameter was conducted. From 167 enriched pathways, oxidative phosphorylation (oxphos) was the only FDR-significantly enriched pathway in the set of 216 positively correlating probe sets (FDR=0.0008). The 173 caloric-responsive probe sets that correlate positively with HDL were also significantly enriched in the oxphos pathway (FDR=0.002).

3.4. Multivariate analyses of caloric-responsive genes

Multivariate analyses were conducted with the caloric-responsive probe sets on the 42 samples composed of 14 subjects and 3 caloric doses of the high-fat meal in order to evaluate the individual impact of the caloric dosing on gene expression in the blood cells of both population groups. First, we carried out an unsupervised PCA with iAUC data (Fig. 3A) as well as with the expression data for each time point, that is, with 126 samples (Suppl. Fig. 1) of the 624 caloric-responsive probe sets. The first principal component (PC1) of the model computed with iAUC data in Fig. 3A explains 40% and second principal component (PC2) 15% of the variability. As the PCA score plot indicates, the samples align along the PC2 according to their metabolic status (NW and OB subjects). Furthermore, the postprandial gene expression of the OB subjects appears more heterogeneous in comparison to the NW subjects, who cluster closer together. However, we also observed a segregation of the samples in two major regions along the PC1 axis. The six right-most points on the PC1 axis represent the postprandial response of three OB individuals to the caloric dose of 1500 kcal and of one OB individual (subject 29) to each of the three caloric doses. An almost identical pattern emerged with the data from the individual time points (see Suppl. Fig. 1) validating the ability of iAUC to reflect postprandial changes in gene expression. These findings motivated an investigation of a potential functional segregation of the "outlier group," which from now on is referred to as the *metabolically active* (MA) group, from the main population group, which is referred to as *metabolically normal* (MN) group. In order to identify genes whose expression differed the most between the two newly generated groups, we conducted a supervised OPLS-DA with the iAUC of the 624 caloric-responsive probe sets and observed a clear separation of the MA and MN groups (Fig. 3B). The degree of variation explained by the OPLS-DA model (R^2) is 0.81, and the value explaining the models predictive ability (Q^2) is 0.78. The model is significant based on the cross validation–analysis of variance technique (CV-ANOVA, $P<.001$).

3.5. Functional analysis of caloric-responsive genes discriminating MN from MA and NW from OB individuals

To conduct a functional analysis of probe sets discriminating MN from MA individuals, we first calculated the VIP-values of each caloric-

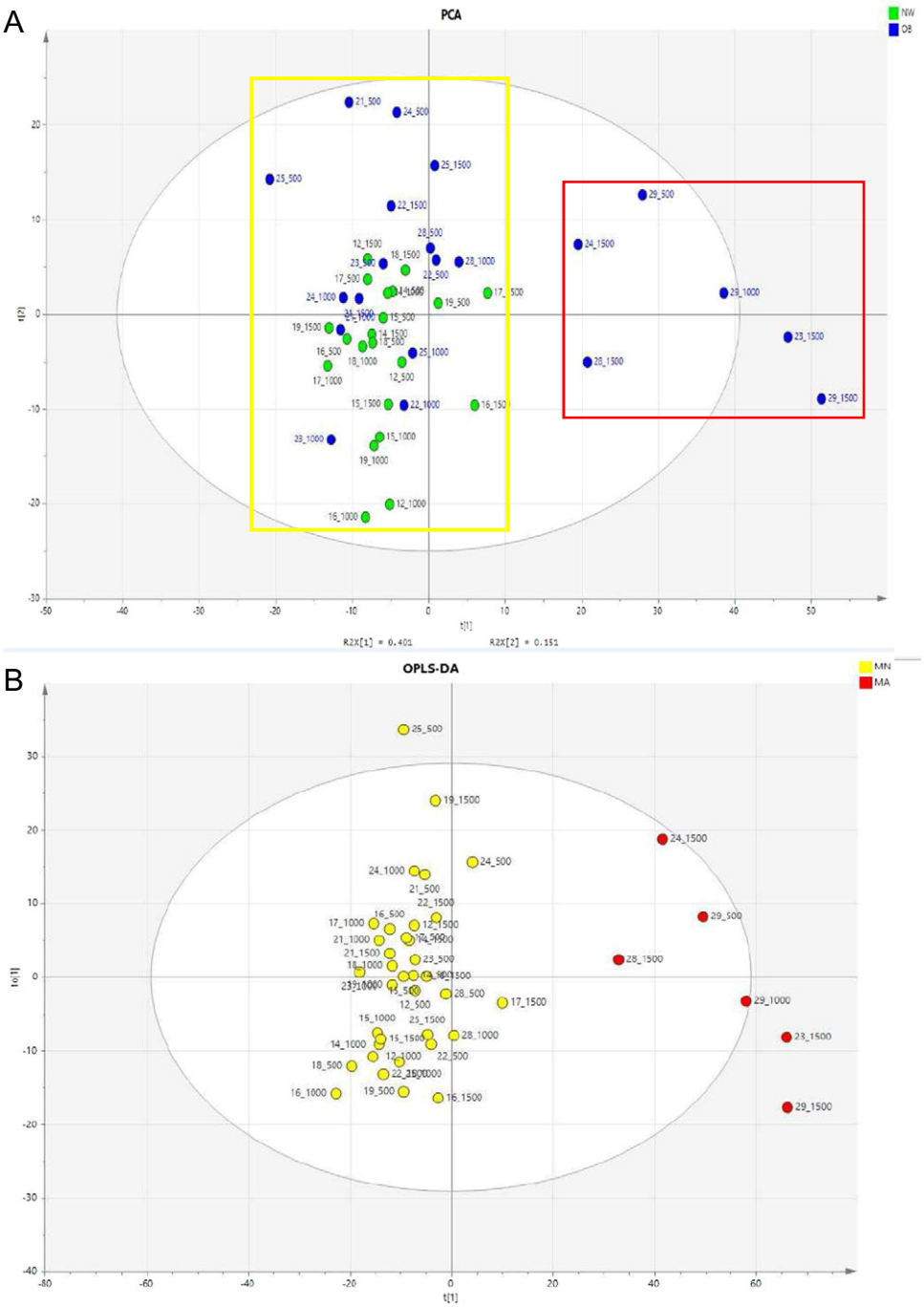

Fig. 3. Multivariate analyses conducted with the 624 caloric-responsive probe sets and all of the 42 samples (14 subjects, 3 caloric doses). Fig. 3A shows the unsupervised PCA score plot with the two first principal components plotted on the x- and y-axis computed with iAUC data of the 624 caloric-responsive probe sets. Data points indicate the subject and meal condition. Green data points belong to the NW group, and blue ones to OB subjects. Color-framed boxes point at a separation of samples in two groups: on the left (yellow frame) the metabolically normal (MN) subjects and on the right (red frame) the metabolically active (MA) subjects. Fig. 3B represents a supervised OPLS-DA score plot also conducted with the iAUC of the 624 caloric-responsive probe sets. The yellow color represents the group of MN subjects and, the red data points, the MA subjects.

responsive probe set from Fig. 3B. In total, 247 probe sets with a VIP-value>1.0, ranging between 2.41 and 1.01, were selected for functional analysis. Again, oxphos appeared, with 13 from 77 probe sets, as the pathway with the highest level of significance (FDR=0.0002), the following eight genes represented by the probe sets being significantly increased: *NDUFB1*, *NDUFB3*, *NDUFS4*, *NDUFS5*, *NDUFA4*, *UQCRB*, *COX6C* and *ATP5I*. The second pathway with the highest statistical significance was ubiquinone metabolism (FDR=0.0086), which shares five caloric-responsive genes with the oxphos pathway. The 13 caloric-responsive probe sets of the oxphos pathway differentiating MA from MN individuals are compiled in Table 2. Furthermore, we included the mean iAUC of each of the 13 probe sets as well as the standard error of the mean (S.E.M.) for the group of 10 MN individuals and four MA subjects. The iAUC of the MN group was negative for nine (iAUC between −0.026 and −0.886) and positive for four probe sets (iAUC between 0.020 and 0.461), seven of them being significantly different from 0 ($P \leq .05$), thus demonstrating a postprandial activity. In the MA group, all 13 probe sets had positive iAUC ranging from 3.540

Table 2
Characteristics of the caloric-responsive probe sets of the oxphos pathway differentiating MA from MN individuals including the Affymetrix ID, gene symbol, mean iAUC and standard error of the mean (S.E.M.) of each probe set for the MN and MA subjects

Affymetrix ID	Gene symbol	MN group (n=36)		MA group (n=6)	
		Mean iAUC	S.E.M.	Mean iAUC	S.E.M.
11715889_a_at	ATP5I	−0.467 *	0.20	3.550 *	0.49
11715890_x_at	ATP5I	−0.388 *	0.22	4.262 *	0.53
11754444_x_at	COX6C	−0.634 *	0.27	7.729 *	0.80
11717634_a_at	COX6C	−0.371	0.28	7.765 *	0.78
11754079_s_at	NDUFA5	0.461 *	0.16	4.993 *	0.91
11721296_a_at	NDUFB1	0.170	0.18	5.599 *	0.78
11764061_s_at	NDUFB3	0.326	0.21	5.419 *	0.70
11717159_a_at	NDUFB3	−0.406	0.25	5.751 *	0.72
11716340_a_at	NDUFS4	−0.602 *	0.27	4.461 *	0.42
11757665_x_at	NDUFS5	−0.784 *	0.24	3.540 *	0.38
11762275_x_at	UQCRB	−0.026	0.13	4.530 *	0.79
11751680_a_at	UQCRB	0.020	0.12	5.826 *	1.10
11716469_x_at	UQCRB	−0.886 *	0.49	10.635 *	1.28

* iAUC values of probe sets were tested to be significantly different from 0 with Wilcoxon-signed rank test (P≤.05).

to 10.635, all of them being significantly different from 0 (P≤.05). In agreement with the results of the multivariate analyses, the regulation of genes such as *UQCRB*, *COX6C* and *ATP5I* differed strongly between individuals (Suppl. Fig. 2), especially among the OB subjects: subjects 24, 23 and 28 showed a larger increase in the expression of these genes after the consumption of 1500 kcal, in comparison to the response after 500 kcal and 1000 kcal; subject 29 exhibited a high activation of these genes after the ingestion of each of the three caloric doses. The other three OB subjects behaved similarly to the seven NW subjects.

To extend the functional analysis beyond the OPLS-DA analysis of the MN and MA subjects, we have conducted a pathway analysis of the caloric-dependent probe sets significantly present in each of the NW and OB groups ($NW_{1500-1000}$: 24 up and 14 down; $NW_{1500-500}$: 28 up and 19 down; $NW_{1000-500}$: 30 up and 72 down; $OB_{1500-1000}$: 132 up and 33 down; $OB_{1500-500}$: 225 up and 86 down; $OB_{1000-500}$: 26 up and 112 down). With the exception of the oxphos pathways for the comparison $OB_{1500-500}$ (8 up-regulated genes: ATP5I, COX VIc, NDUFA5, NDUFB, NDUFB3, NDUFS4, NDUFS5, UQCRB; FDR=0.0005) and the comparison $OB_{1500-1000}$ (4 up-regulated genes: COX VIc, NDUFB3, NDUFS5, UQCRB; FDR=0.046) as well as the related pathway "ubiquinone metabolism" for the comparison $OB_{1500-500}$ (5 up-regulated genes: NDUFA5, NDUFB1, NDUFB3, NDUFS4, NDUFS5; FDR=0.004) no significant pathways with more than three differentially expressed genes were observed.

Among the 624 caloric-responsive probe sets, only few were also identified as being part of one of the three major BMI-dependent functional gene expression signature identified in whole blood by Homuth *et al.* [21], namely, attenuated insulin signaling (7 from 251 genes), erythrocyte to reticulocyte ratio shift (14 from 168 genes) and reduced defense against oxidative stress (2 from 62 genes). In addition, a box plot of the distribution of the iAUCs of the genes belonging to each of these three expression signatures did not reveal differences in their distribution throughout the six groups of the NW and OB subjects (NW_{500}, NW_{1000}, NW_{1500}, OB_{500}, OB_{1000} and OB_{1500}) and well as throughout the six groups of the MN and MA subjects (MN_{500}, MN_{1000}, MN_{1500}, MA_{500}, MA_{1000} and MA_{1500}) (data not shown).

3.6. CellMix function for estimating blood cell composition

In order to estimate the postprandial changes in blood cell composition in NW and OB subjects, we applied the CellMix function published by Gaujoux and Seoighe [22]. Fig. 4 shows the CellMix plot predicting the blood cell type proportions (based on cell type-specific gene signatures). The composition does not appear to be affected by BMI, since there is no major difference between samples from obese and normal weight subjects. All samples are close to the expected "normal" cell-type composition (red lines in each plot), except for some samples of subject 29, who was anyhow in a different metabolic status than the other subjects (as already mentioned in Section 3.5). Fig. 4 clearly indicates that the interindividual difference in cell distribution is the most variable parameter, being high in comparison to intraindividual differences. Finally, we could not observe significant postprandial changes (P>.05) in blood cell composition by applying the CellMix test on samples from NW and OB subjects or samples from MN and MA subjects.

4. Discussion

Nutrient excess is associated with insulin resistance as well as with inflammation and obesity[24]. In that context, Homuth *et al.* reported attenuated insulin signaling and reduced defense against oxidative stress with increasing BMI in the whole blood transcriptome of fasting subjects [21]. In addition, consumption of a Mediterranean diet as well as a diet rich in monounsaturated fatty acids, compared with a diet rich in saturated fatty acids, decreased the expression of oxphos genes in peripheral blood mononuclear cells of abdominally overweight subjects [25]. These reports suggest that transcriptomic profiling could also be used to monitor the postprandial impact of food ingestion on cellular metabolism using blood cells as a model system [14]. In addition, applying a caloric dose–response challenge to the human organism may allow to better differentiate subtle difference in metabolic fitness of individuals [13]. We have therefore explored the postprandial transcriptome of 168 whole blood samples contributed by seven NW and seven OB male subjects before (0 h) and 2, 4 and 6 h after the consumption of three caloric doses of a high-fat meal. Our study represents the first genome-wide transcriptomic dose-dependent analysis of the response of the human organism to increasing caloric doses of a meal.

Caloric-dependent changes in gene expression could clearly be observed in blood cells in response to the high-fat meal in NW and OB subjects. In particular, the intensities and number of significantly expressed genes differed according to the metabolic status of the subjects and the caloric dose that they consumed. We have identified 624 caloric-responsive probe sets that react in a quantitative manner to the increase in the caloric dosing of the high-fat meal. Out of the six conditions tested (iAUCs of NW_{500}, NW_{1000}, NW_{1500}; OB_{500}, OB_{1000}, OB_{1500}), the OB subjects responded with the largest changes in expression of these genes after the ingestion of 1500 kcal showing that the metabolic status of the subjects is correlated with their postprandial response to increasing caloric doses of the high-fat meal. That NW subjects react differentially to the three caloric doses in comparison to OB subjects was also highlighted by the classical clinical chemistry parameters for the main cohort of this randomized crossover intervention study [12]. The initial trial studying the postprandial responses of the main cohort (19 NW subjects, 17 OB subjects) revealed significant caloric dose-dependent differences for some metabolic, inflammatory and hormonal parameters in both groups. However, the only variable that could differentiate the postprandial response of normal weight and obese subjects at each of the three caloric doses of the high-fat meal was insulin [12].

Out of the 624 caloric-responsive probe sets, one third correlated positively with the postprandial insulin response in the OB subjects, whereas gene expression of NW subjects showed hardly any significant correlation with this hormone. This finding suggests that the postprandial blood cell transcriptome and postprandial serum clinical chemistry are linked to each other in individuals with impaired metabolism. The correlation patterns of Fig. 2 further highlight the heterogeneous responsive character of the individuals belonging to

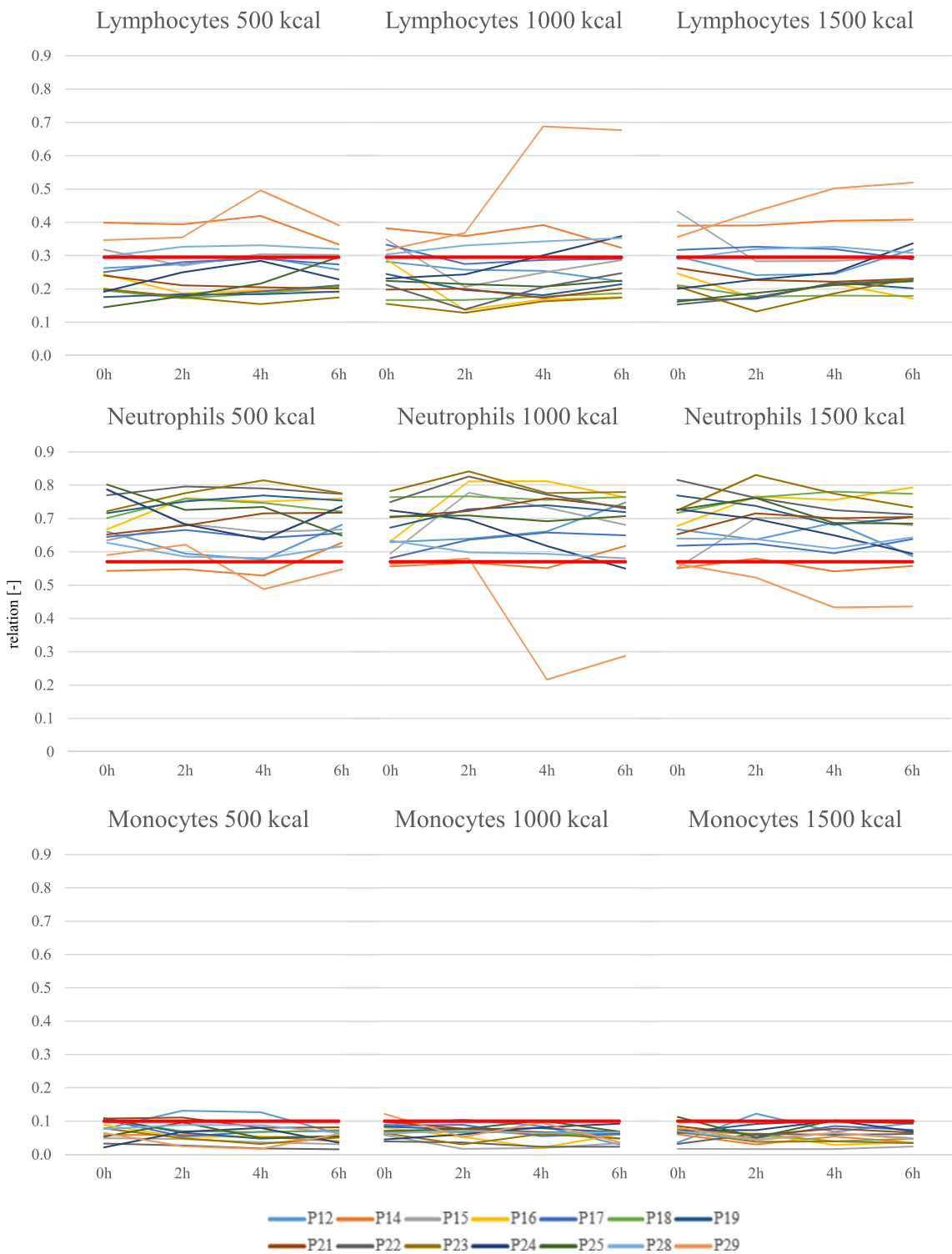

Fig. 4. Predicted distribution of lymphocytes, neutrophils and monocytes in the fasting and postprandial blood samples after ingestion of each of the three caloric doses of the high-fat meal. The x-axis represents the time postingestion (0–6 h). The y-axis gives the predicted proportion of cells using the CellMix algorithm. The red line indicates the reference relation of the corresponding blood cell type. Each color-coded line represents the kinetic behavior of a NW subject (P12-P19) or an OB subject (P21-P29).

the two groups of subjects. The NW subjects showed weaker correlations than the OB subjects between clinical chemistry parameters and gene expression. That the postprandial transcriptome of the OB subjects reacted less homogenously to the caloric challenges was indicated by the analysis of variance of the 624 selected probe sets. Taken together, our data indicate that the obese individuals — although all of them were metabolically healthy based on their clinical chemistry parameters at baseline — respond differently at a molecular level that can be highlighted with the postprandial gene expression signature of their blood cells. In order to follow up that assumption we used the caloric-responsive probe sets being positively and negatively associated with insulin for functional analyses. The caloric-responsive

genes that were positively correlated with postprandial insulin were also highly significantly enriched in the oxphos pathway. This pathway is a central part of human energy metabolism being responsible for the energy production (ATP) in mitochondria. A permanent excess of nutrients leads to the dysfunction of mitochondria, which in turn causes obesity-related pathologies probably due to the negative effects of reactive oxygen species [26]. A disturbed activity of the oxphos pathway in fasting samples of OB subjects has already been identified in the fasting whole blood transcriptome [27] and furthermore in other tissues such as liver [28], peripheral blood mononuclear cells [25], adipose tissue [26] and skeletal muscle [5,29]. However, data were not in agreement, depending on the type of meal or diet administered as well as the health status of the study participants (*e.g.*, insulin-resistant individuals) [25,27]. In our study, we showed that the oxphos pathway was activated postprandially in a manner that is dependent on the metabolic status of the subjects and the amount of consumed calories, suggesting an interaction between these two components. However, this metabolic status is not solely defined by the BMI of the subjects — our multivariate analyses showed different individual postprandial responses of the OB subjects to the increasing caloric doses of the high-fat meal. In particular, the consumption of 1500 kcal by the OB subjects led to a separation of this group into "outliers" [metabolically active (MA)] and the other individuals, who responded to the high-fat meal in a manner that was similar to the "metabolically normal" (MN) group of NW subjects.

Esser *et al.* [30] have showed that the blood cell composition changes after the consumption of proinflammatory high-fat drinks (88% energy from fat) in lean and obese subjects. In particular, the relative cell count of neutrophils, which represent the major white blood cell population with relative cell counts ranging between 65 and 72%, increased by 6–10% 4 h after ingestion of the drinks. In addition, the metabolic status of the subjects can be one possible factor affecting gene-expression *via* alteration of the blood cell composition as suggested by Homuth *et al.* [21] in the form of a BMI-dependent shift in the erythrocyte-reticulocyte ratio. The postprandial transcriptome of whole blood might thus be dependent on its cell composition. A limitation in our study is that we did not count the blood cells after the consumption of the meals to directly evaluate the impact of such changes on the measured gene expression program. Using a range of cell-specific gene expression signatures, we have addressed, in our gene expression dataset, the presence of postprandial changes in cell distribution in the normal weight and obese groups of subjects. However, we have obtained no evidence for such effects. Furthermore, the 624 caloric-responsive probe sets discussed in this report were selected based on the postprandial dose-dependent increase or decrease by comparing the changes in iAUC of each subject. This approach should minimize the impact of postprandial fluctuations in blood cell composition on the overall transcriptome as only differential changes resulting from the increase in caloric dose should influence the overall blood cell transcriptome. Taken together, although we could not exclude that postprandial changes in cell distribution do take place in our dataset, changes in gene expression is the most likely explanation for our findings.

We hypothesize that this difference between the MA and MN groups might be explained by control mechanisms with negative feedback activity which impairs the digestion or absorption of large amounts of macronutrients in MN individuals and which may be less efficient in the MA individuals [31–34]. With this hypothesis, the postprandial nutrient composition of blood might be differentially altered in the MA individuals in comparison to MN subjects, which may in turn differentially effect gene expression in the blood cells. Alternately, the ability of the blood cells to postprandially respond to the same nutrient content may differ due to individual intrinsic differences in the metabolic fitness of these cells [10]. In line with both hypotheses, we assume that the difference in behavior of the caloric-responsive genes highlights a shift from protective mechanisms involving metabolic saturation in MN individuals to a loss of these control mechanisms in MA individuals [35–37]. Interestingly, the postprandial kinetics of expression of the caloric-responsive genes associated with the oxphos pathway do not indicate a return to fasting values at 6 h in the MA individuals, in particular after ingestion of 1500 kcal of the high-fat meal. It is therefore interesting to speculate that the expression of caloric-responsive genes in blood cells of subjects exposed to a high-fat challenge can be used as a biomarker to identify individuals with an impaired metabolic flexibility [3,5,11].

One special case in the MA group was Volunteer 29, who not only responded "actively" after consuming 1500 kcal of the high-fat meal but also after 500 and 1000 kcal. In addition, the clinical parameters revealed anomalies compared to other obese subjects for what reason this subject was excluded from the data analysis of clinical chemistry parameters [12]. We hypothesize that the regulation of the caloric-responsive genes highlights a subclinically impaired insulin sensitivity [21,27].

DeJong *et al.* [38] as well as Olza and Calder [13] highlighted the importance of dose–response studies in nutrition research. In analogy to pharmacology, such study designs take into account thermodynamic processes characterizing the interaction of nutrients with their receptors. By integrating a kinetic component into our study, we have introduced one additional dimension to the dose–response relationship allowing us to further characterize this interaction. Indeed, the caloric dose dependency as well as the variability in the kinetic patterns observed in our study indicates that these parameters should be carefully considered while investigating the mechanisms taking place during the acute response of the organism to the ingestion of food, even more when subjects with different metabolic status are studied. If not considered, the conclusions that could be extrapolated from more restricted study designs lacking the dose–response and kinetic dimensions could risk validity of their conclusions [12].

Despite the limited number of subjects investigated in our study, our analytical "matrix" strategy combining a kinetic postprandial analysis of the blood cell transcriptomic response of two metabolic groups of subjects to three caloric doses of the high-fat meal in a crossover design allowed us to identify 624 statistically significant caloric-responsive probe sets, as well as biologically meaningful functional links to the oxphos pathway and insulin. The absence of significant pathways apart from "oxidative phosphorylation" in the 624 caloric-dependent probe sets is striking. This lack of additional differentially expressed pathways may result from a research deficit in the literature on functional aspects related to the postprandial response of blood cells. On the other hand, most of the changes in gene expression that take place under postprandial conditions in blood cells appear to be of small magnitude so that many of these pathways may be missed when the cutoff values for the selection of the differentially expressed genes are set relatively high, as it was done in this report. At the same time, our finding highlights the potential of the oxphos pathway in blood cells as a robust biomarker for the quality of the postprandial response of the human organism to the ingestion of meals. In light of the limited number of subjects in both groups, the validity of these conclusions for broader populations should be questioned, and the results should be replicated in other studies. Nonetheless, Ghosh *et al.* [27] also identified the oxphos pathway as being differentially expressed in the blood cell transcriptome of lean and obese subjects supporting the validity of our finding.

Transcriptomic analytical strategies offer the enormous advantage of allowing, in a single measure, a genome-wide semiquantitative evaluation of the reactivity of the entire cellular genome in response to a stimuli, in our particular case, a dietary challenge. On the other hand, the posttranscriptional process is complex, and gene expression is only indicative of potential changes at the protein and metabolite levels. In particular, genes of the oxphos pathway mostly code for

enzymes, and their protein abundance and enzymatic activity are not always concordant with gene expression. In that context, and despite its limitation in the identification of metabolites, metabolomics is a powerful approach to complement gene expression data with downstream data along the cellular flow of information. Further, integrating transcriptomic and metabolomic data provides holistic insights into biological processes as shown by Bartel *et al.* [39] for the human blood transcriptome and serum metabolome. Consequently, we are currently conducting metabolomic analyses of the samples of this study with liquid and gas chromatography–mass spectrometry (LC–MS and GC–MS).

In summary, we observed an altered postprandial transcriptomic regulation in the blood cells of OB subjects compared to NW subjects after increasing caloric doses of a high-fat meal challenge. This response was associated with postprandial insulin and with the oxphos pathway. A more detailed analysis of the data allowed us to separate individuals in the group of OB subjects into individuals whose response was close to the NW subjects and a second group of individuals with a transcriptional response, which might be indicative of a loss of metabolic flexibility, in particular after consumption of the highest caloric dose. We propose that the molecular signature provided by the postprandial transcriptomic response of blood cells to a meal challenge provides a sensitive and physiologically meaningful way to evaluate the qualitative impact of food on human health [12,13].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2017.02.012.

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